Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus

Review

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TGF-β signaling controls a plethora of cellular responses and figures prominently in animal development. Recent cellular, biochemical, and structural studies have revealed significant insight into the mechanisms of the activation of TGF-β receptors through ligand binding, the activation of Smad proteins through phosphorylation, the transcriptional regulation of target gene expression, and the control of Smad protein activity and degradation. This article reviews these latest advances and presents our current understanding on the mechanisms of TGF-β signaling from cell membrane to the nucleus.

Introduction

Transforming Growth Factor β (TGF-β) signaling controls a diverse set of cellular processes, including cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate, during embryogenesis as well as in mature tissues, in species ranging from flies and worms to mammals (Patterson and Padgett, 2000; ten Dijke et al., 2002; Massagué et al., 2000).

A TGF-β ligand initiates signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase domain, which then propagates the signal through phosphorylation of the Smad proteins (Figure 1). There are eight distinct Smad proteins, constituting three functional classes: the receptor-regulated Smad (R-Smad), the Co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads (Smad1, 2, 3, 5, and 8) are directly phosphorylated and activated by the type I receptor kinases and undergo homotrimerization and formation of heteromeric complexes with the Co-Smad, Smad4 (Figure 1). The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes. The I-Smad, Smad6 and Smad7, negatively regulate TGF-β signaling by competing with R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation (Figure 1).

Because TGF-β signaling generally has a negative effect on cell growth, inactivation of this pathway contributes to tumorigenesis. Tumor-derived mutations

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have been observed in both TGF-β family receptors and the Smad proteins. The TGF-β type II receptor is inactivated by mutation in most human gastrointestinal cancers with microsatellite instability and Smad4 in nearly half of all pancreatic carcinomas. Many other somatic and hereditary disorders are a result of mutations or malfunctions in the TGF-β pathway (reviewed in Massagué et al., 2000). Naturally occurring disease mutations in Smads and TGF-β family receptors have enormously facilitated the analysis of the structure and function of these proteins.

Over the last decade of intense investigation, many key events in TGF-β signaling have been documented at the molecular and cellular level. An earlier focus on the cell biological characterization has now been complemented by biochemical and structural investigation, giving rise to an unprecedented level of clarity in several aspects of the signal transduction process. In this review, we focus on the molecular mechanisms of TGF-β signaling and present a comprehensive picture by synthesizing the known cellular, biochemical, and structural features of the TGF-β pathway common to all cell types in organisms from fruit fly to human.

TGF-β Ligands and Receptors

The TGF-β family of cytokines, characterized by six conserved cysteine residues, are encoded by 42 open reading frames in human, 9 in fly, and 6 in worm (Lander et al., 2001). It contains two subfamilies, the TGF-β/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Muellerian inhibiting substance) subfamily, as defined by sequence similarity and the specific signaling pathways that they activate (Figure 2).

Although the diverse TGF-β ligands elicit quite different cellular responses, they all share a set of common sequence and structural features. The active form of a TGF-β cytokine is a dimer stabilized by hydrophobic interactions, which are further strengthened by an intersubunit disulfide bridge in most cases (Figure 3A). Each monomer comprises several extended β strands interlocked by three conserved disulfide bonds that form a tight structure known as the "cysteine knot" (Sun and Davies, 1995). The dimeric arrangement of the ligands suggests the formation of a complex with two type I and two type II receptors. Ligand access to the receptors is regulated by a large family of proteins collectively known as ligand traps (Figure 2). The crystal structure of the Noggin-BMP7 complex reveals that the ligand trap Noggin inhibits BMP7 by blocking the surfaces that are required to interact with the type I and type II BMP receptors (Groppe et al., 2002) (Figure 3B).

The receptor serine/threonine kinase family in the human genome comprises 12 members—7 type I and 5 type II receptors—all dedicated to TGF-β signaling (Manning et al., 2002) (Figure 2). Both types of the receptor serine/threonine kinases consist of about 500 amino acids, organized sequentially into an N-terminal extracellular ligand binding domain, a transmembrane region,
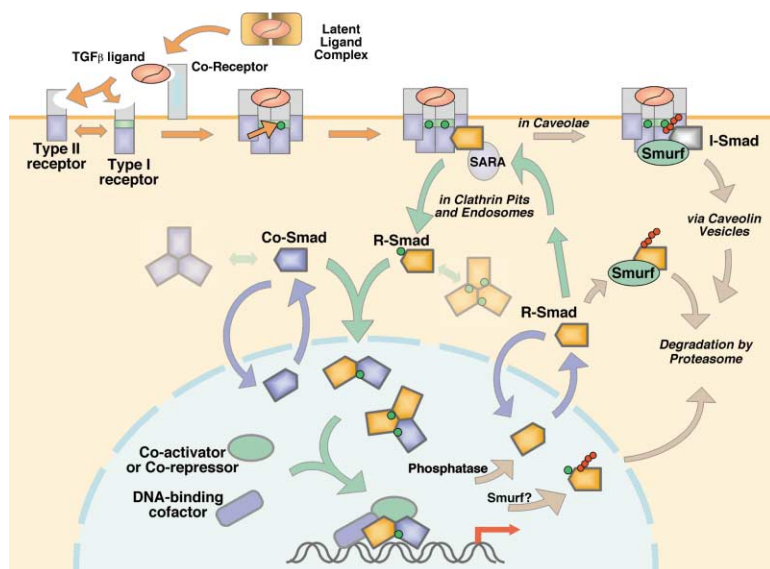
Figure 1. Schematic Diagram of TGF-β Signaling from Cell Membrane to the Nucleus

The arrows indicate signal flow and are color coded: orange for ligand and receptor activation, gray for Smad and receptor inactivation, green for Smad activation and formation of a transcriptional complex, and blue for Smad nucleocytoplasmic shuttling. Phosphate groups and ubiquitin are represented by green and red circles, respectively.

and a C-terminal serine/threonine kinase domain. The overall structures of the extracellular ligand binding domain of the type I BMP receptor (Kirsch et al., 2000) as well as the type II receptors for Activin (Greenwald et al., 1999) and TGF-β (Boesen et al., 2002) exhibit a similar three-finger toxin fold, with each finger formed by a pair of anti-parallel β strands (Figure 4A).

The type I, but not type II, receptors contain a characteristic SGSGSG sequence, termed the GS domain, immediately N-terminal to the kinase domain. The activation of the type I receptor involves the phosphorylation of its GS domain by the type II receptor; hence an active receptor signaling complex comprises both types of receptors bound to the ligand. Several receptor variants have N-terminal or C-terminal extensions, most of them with as yet unknown function (Massagué, 1998). Truncating mutations of a long C-terminal extension of the human BMP type II receptor (BMPR-II) appear to cause

Figure 2. A Schematic Relationship Describing TGF-β Ligands, Ligand Binding Traps, Accessory Receptors, and the Type I and II Receptors in Vertebrates

The downstream R-Smads 1, 2, 3, 5, and 8 are grouped based on their signaling specificity. Commonly used alternative names are: ALK2/ActR-I, ALK3/BMPR-IA, ALK4/ActR-IB, ALK5/TβR-I, and ALK6/BMPR-IB.

the hereditary disorder primary pulmonary hypertension (De Caestecker and Meyrick, 2001). These mutants still appear normal in their ability to activate Smads via BMP type I receptors (Nishihara et al., 2002), raising the possibility that the BMPR-II tail has a critical, Smad-independent signaling function.

Mechanism of Ligand Binding to the Receptors

Two distinct modes of the ligand-receptor interaction exist, one exemplified by members of the BMP subfamily and the other represented by TGF-βs and Activins. BMP ligands such as BMP2 and -4 exhibit a high affinity for the extracellular ligand binding domains of the type I BMP receptors and a low affinity for the type II receptors. The preassembled type I receptor-ligand complex has a higher binding affinity for the type II receptor. The structure of the homodimeric BMP2 in complex with two ectodomains of the BMP type I receptor (BMPR-IA) reveals that the receptor binds to the "wrist" epitope of the BMP2 dimer and makes extensive contacts to both BMP2 monomers (Kirsch et al., 2000) (Figure 4B). These interactions are predominantly hydrophobic, with Phe85 of BMPR-IA playing a key role. The aromatic side chain of Phe85, with knob-into-hole packing, points into a hydrophobic pocket formed at the interface of the two BMP2 monomers. Phe85 is preserved or replaced by a similar residue in all type I receptors except ALK1; all residues that form the hydrophobic pocket in the dimeric BMP2 are also highly conserved in all other TGF-β ligands. This suggests that the hydrophobic knob-and-pocket binding is essential for the interactions between TGF-β ligands and the type I receptors (Kirsch et al., 2000).

In contrast to the BMPs, TGF-β and Activin display a high affinity for the type II receptors and do not interact with the isolated type I receptors (Massagué, 1998). In this case, the ligand binds tightly to the ectodomain of the type II receptor first; this binding allows the subsequent incorporation of the type I receptor, forming a large ligand-receptor complex involving a ligand dimer
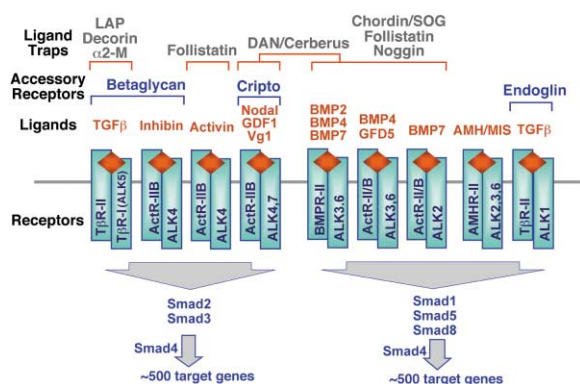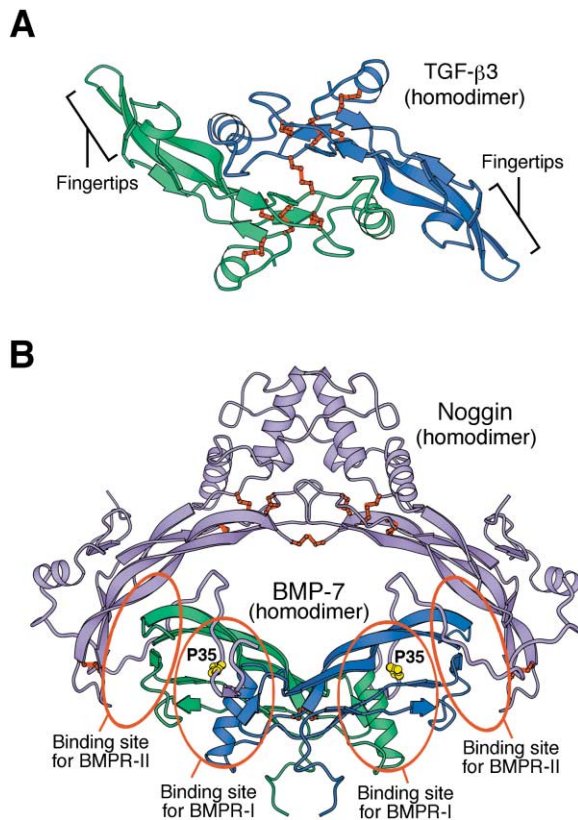
Figure 3. TGF-β Ligand and a Ligand Binding Trap

(A) Structure of a representative TGF-β ligand, TGF-β3 (Mittl et al., 1996). The two monomers are colored blue and green, respectively. Cysteine side chains and disulfide bonds are represented by red lines. Note the dimeric ligand is linked by a disulfide bond.

(B) Structure of a Noggin dimer (colored purple) in complex with a BMP-7 homodimer. The N-terminal fragments of Noggin interact with the same surface areas of BMP-7 as required for binding to the type I and II receptors. A critical residue Pro35 of Noggin is shown in yellow. This residue occupies the same hydrophobic pocket of BMP-7 as required for binding to Phe85 of the type I BMP receptor. Figures 3, 4, 5, 6, and 8 were prepared using MOLSCRIPT (Kraulis, 1991).

and four receptor molecules. Structural analysis of the ectodomain of the human TGF-β type II receptor (TβRII) in complex with TGF-β3 reveals that the binding occurs at the far ends (so-called "fingertips") of the elongated ligand dimer (Hart et al., 2002) (Figure 4C). Each receptor only binds to one monomer of the dimeric TGF-β3. This interaction creates two symmetrically positioned concave surface patches, which were postulated to be the binding site for the ectodomain of the type I receptor (Figure 4C).

Although the interactions between the type I receptors and their associated ligands appear to be conserved for all TGF-β family members, significant variation exists for the binding between the different subfamilies of the type II receptors and their ligands (Gray et al., 2000; Hart et al., 2002). The Activin type II receptor has a broad specificity and can bind to both Activin and BMP ligands. Compared to TβRII, the ectodomain of the Activin type II receptor (ActRII) contains a quite different ligand binding epitope, as documented in its cocrystal
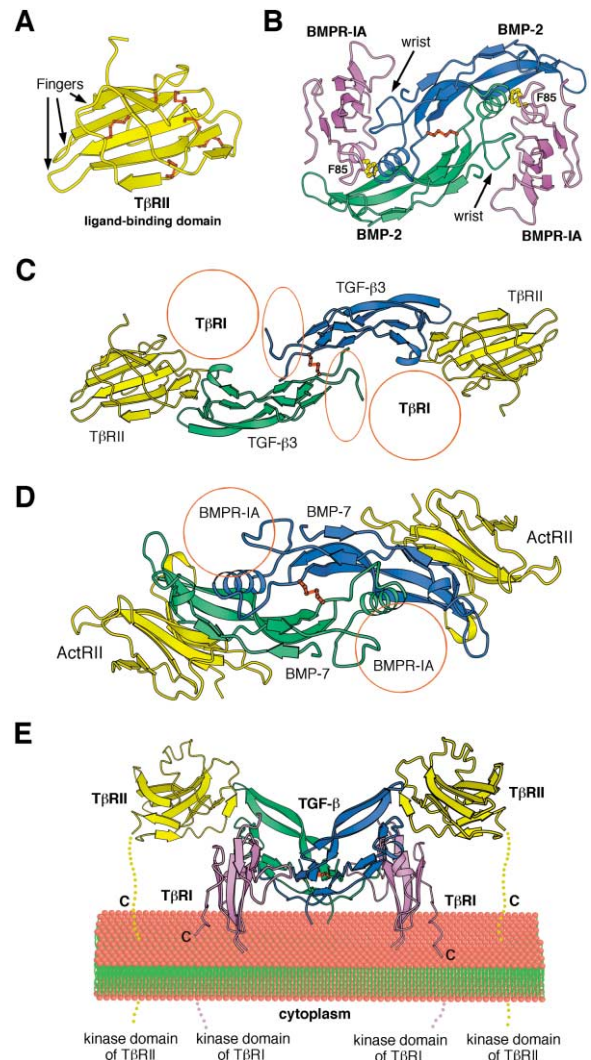

Figure 4. Assembly of the Ligand-Receptor Complexes

(A) Structure of the extracellular ligand binding domain of the type II TGF-β receptor (Boesen et al., 2002; Marlow et al., 2003). The structure resembles the so-called three-finger toxin fold (Greenwald et al., 1999).

(B) Structure of the extracellular ligand binding domain of the type IA BMP receptor bound to BMP2 (Kirsch et al., 2000). The highly conserved residue in type I receptors (Phe85 in BMPR-IA) is shown in yellow.

(C) Structure of the extracellular ligand binding domain of the type II TGF-β receptor bound to TGF-β3 (Hart et al., 2002). The disordered "wrist" regions of the BMP2 dimer are indicated by red ovals whereas the predicted TβRI binding sites are shown by red circles. The orientation of TGF-β3 is the same as BMP2 in (B).

(D) Structure of the extracellular ligand binding domain of the type II Activin receptor bound to BMP7 (Greenwald et al., 2003). Compared to TβRII-TGFβ3, ActRII uses a different ligand binding epitope to contact a different surface on BMP7. The orientation of BMP-7 is related to that of BMP2 (B) by a clockwise 50° rotation along an axis perpendicular to the paper.

(E) A structural model of the complete extracellular TGF-β receptor complex. The structure of the BMPR-IA was used to model the extracellular ligand binding domain of the type I receptor of TGF-β receptor. The modeled structure is viewed along the plasma membrane. The C termini of the receptors are represented by dashed lines going into the plasma membrane.

structure with either BMP7 (Greenwald et al., 2003) (Figure 4D) or with Activin A (Thompson et al., 2003). Sequence analysis suggests that MIS and AMHR-II may use a third unique binding interface (Greenwald et al., 2003), further highlighting the complexity in ligand binding to the type II receptors. These observations identify different modes of receptor complex assembly and support the idea that the type II receptors play a more important role in the determination of specificity for the assembly of the receptor signaling complex.

Two contrasting models have been proposed to explain the two-step assembly of a functional signaling complex for TGF-βs and Activins. In the allosteric model, the binding of ligand to the type II receptor is required to induce a conformational change in the ligand, which leads to exposure of the binding epitope for the type I receptor. Supporting this model, the TβRII bound TGF-β3 does undergo a large conformational change compared to its uncomplexed form (Hart et al., 2002) (Figures 3A and 4C). In the cooperative model, the ectodomain of the type I receptor interacts with an extended surface that relies on the formation of the type II receptor-ligand complex. The cooperative model was thought to apply to the assembly of TGF-β ligand-receptor complex based on modeling studies (Hart et al., 2002). However, the structure of ActRII-BMP7, together with that of the BMPRIA-BMP2 complex (Kirsch et al., 2000), allows the molecular construction of a model for the BMP7-ActRII-BMPRIA complex (Greenwald et al., 2003). In this model, the ectodomains of the type I and type II receptors do not directly contact each other; yet these two receptors bind cooperatively to the BMP7 ligand. Thus this cooperativity is likely derived from an allosteric effect (Greenwald et al., 2003).

Mechanism of Receptor Activation

Binding to the extracellular domains of both types of the receptors by the dimeric ligand induces a close proximity and a productive conformation for the intracellular kinase domains of the receptors, facilitating the phosphorylation and subsequent activation of the type I receptor (Figure 4E). The type II receptor kinases are thought to be constitutively active, although the regulation of this process remains unclear. The type II receptor kinase phosphorylates multiple serine and threonine residues in the TTSGSGSG sequence of the cytoplasmic GS region of the type I receptor, leading to its activation (Massagué, 1998) (Figure 5). Because of its critical role in receptor activation, the GS region serves as an important regulatory domain for TGF-β signaling. For example, the immunophilin FKBP12 inhibits TGF-β signaling by binding to the unphosphorylated GS regions of the type I receptors (Huse et al., 1999). This interaction also locks the kinase catalytic center of the type I receptors in an unproductive conformation (Figure 5).

Significant details on the mechanism of receptor activation were revealed by a recent study in which a TβRI protein homogeneously tetra-phosphorylated in the GS region was generated using an in vitro protein ligation strategy (Huse et al., 2001). This represents the first successful example of chemically creating a phosphorylated molecule to study TGF-β signaling. In contrast to the inability of the unphosphorylated TβRI to interact with its downstream target Smad2, the phosphorylated TβRI protein binds efficiently to Smad2 in vitro and exhibits a dramatically enhanced phosphorylation specificity for the C-terminal serine residues of Smad2 (Huse et al., 2001) (Figure 5). In addition, the tetra-phosphorylated TβRI can no longer be recognized by the inhibitory protein FKBP12. Thus, phosphorylation activates TβRI by switching the GS region from a preferred binding site for an inhibitor into a binding surface for R-Smad substrates.

Regulation of Receptor Activation

The steps leading to receptor activation are tightly regulated. The access of TGF-β ligands to their receptors is controlled by two classes of molecules with opposing function (Figures 1 and 2). One class comprises a diverse group of soluble proteins that act as ligand binding traps, sequestering the ligand and barring its access to membrane receptors. They include the proregion of TGF-β precursor, which after cleavage in the secretory pathway remains noncovalently bound to the bioactive domain as a "latency-associated polypeptide" (LAP). They also include the small proteoglycan decorin and the circulating protein α2-macroglobulin, which bind to free TGF-β; follistatin, which binds to Activins and BMPs; and three distinct protein families—Noggin, Chordin/SOG, and DAN/Cerberus—whose members also bind to BMPs.

Our knowledge of the biological role of these proteins has been extensively reviewed (Balemans and Van Hul, 2002; Harland, 2001; Massagué and Chen, 2000), but until recently our understanding of how these factors block TGF-β ligands was lacking. The resolution of the crystal structure of the Noggin-BMP7 complex has revealed for the first time how one of these factors binds to its target and prevents it from contacting membrane receptors (Groppe et al., 2002). Noggin is a critical regulator of BMP activity during vertebrate dorsal-ventral patterning, neuronal induction and differentiation, and skeletogenesis and joint formation (Brunet et al., 1998; Gong et al., 1999; Lim et al., 2000). Noggin functions as an antagonist of several BMPs, including BMP7. The structure of the Noggin-BMP7 complex reveals that Noggin inhibits BMP7 by blocking the surfaces that are required to interact with the type I and type II BMP receptors (Groppe et al., 2002) (Figure 3B). The N-terminal segment of each Noggin monomer adopts an extended conformation and wraps around a BMP7 monomer, directly occupying the receptor contact sites. A proline residue in this segment, Pro35, fills the BMP7 hydrophobic pocket that normally accommodates Phe85 in the type I receptor. Interestingly, the core structure of the Noggin monomer contains a cysteine knot. This, and the overall structural configuration suggest that ligand and antagonist may have evolved from a common ancestor.

The other class of molecules that control ligand access to receptors includes membrane-anchored proteins that act as accessory receptors, or coreceptors, promoting ligand binding to the signaling receptors. Recent work has revealed that the coreceptors for the TGF-β family play a broader role than previously thought. The membrane-anchored proteoglycan beta-
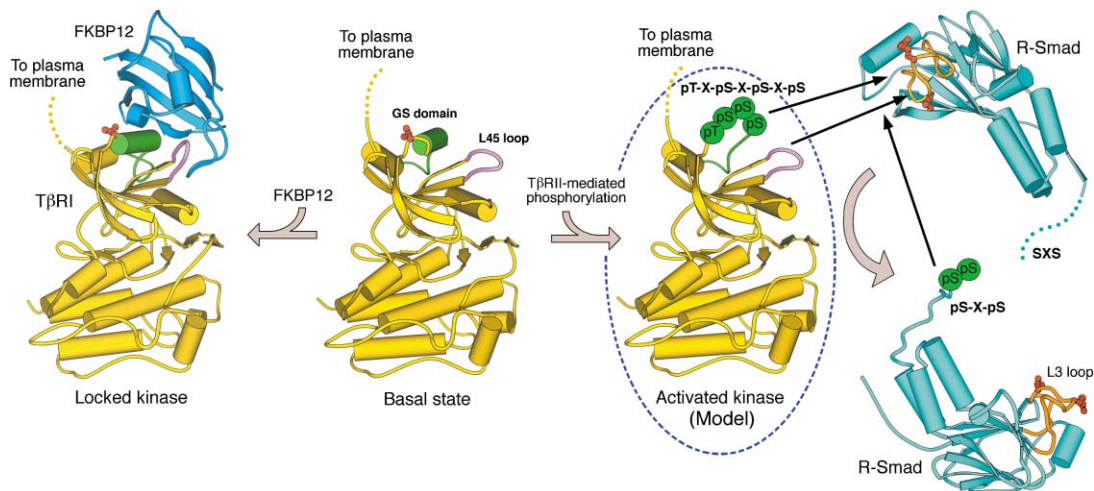
Figure 5. Activation of the Type I Receptor Kinase and Recognition of R-Smad

In the basal state, type I receptor remains unphosphorylated (Huse et al., 2001). This conformation can be recognized either by FKBP12 (Huse et al., 1999) for sequestration in an inactive state (leftward arrow) or by TβRII for phosphorylation in the GS domain (rightward arrow). After phosphorylation, TβRI uses its GS domain and L45 loop to interact with the basic pocket and L3 loop of an R-Smad, resulting in its phosphorylation in the C-terminal SXS motif. The pThr/pSer-X-pSer motif on the R-Smad and the type I receptor is shown as green spheres.

glycan, also known as the TGF-β type III receptor, has long been known to mediate TGF-β binding to the type II receptor, a role that is particularly critical for TGF-β2 (one of three mammalian TGF-βs) (Brown et al., 1999a; Massagué, 1998). Betaglycan does not bind to Activins or BMPs. Recently, however, betaglycan was shown to bind to inhibin and to facilitate its access to Activin receptors, thus outpacing Activin and blocking its action (Lewis et al., 2000). Cripto and other members of the CFC-EGF family, produced both as secreted factors and as cell surface components, mediate the binding of Nodal, Vg1, and GDF1 to Activin receptors (Cheng et al., 2003; Rosa, 2002; Shen and Schier, 2000). In an unusual case of dual function, connective tissue growth factor (CTGF) has been shown to enhance TGF-β binding to its receptors and inhibit BMP4 binding to BMP receptors (Abreu et al., 2002).

Genetic and biochemical evidence suggests that the betaglycan-related protein endoglin facilitates binding of as yet unknown TGF-β family members to the type I receptor ALK1 in endothelial cells, and this role is critical for vascular homeostasis (Marchuk, 1998; Massagué et al., 2000). The ligand in question could be TGF-β itself; recent reports indicate that TGF-β somehow activates ALK1 in endothelial cells, creating a counterbalance between ALK1 signaling via Smad1 and TβR-I signaling via Smad3 (Goumans et al., 2002) (Figure 2).

The protein BAMBI (*B*MP and *A*ctivin receptor *m*embrane *b*ound *i*nhibitor, also known as Nma) represents a very different type of negative regulator of receptor activation (Onichtchouk et al., 1999). BAMBI has structural features of a decoy receptor, with an extracellular domain and a short cytoplasmic region that share sequence similarities with type I receptors. BAMBI competes with the type I receptor for incorporation into ligand-induced receptor complexes, inhibiting receptor activation. During *Xenopus* embryo development, BAMBI expression is induced by BMP, creating a nega-

tive feedback loop that helps ensure a proper level of BMP signaling.

Receptor activation is also regulated by intracellular proteins. Binding of FKBP12 to the unphosphorylated GS domain of type I receptors is thought to constrain the basal activity by preventing ligand-independent phosphorylation and activation of the receptor (Huse et al., 1999). Once activated, the TGF-β family receptors are negatively regulated by the I-Smad, Smad7. Smad7 binds to the activated receptors in competition with R-Smads (Kavsak et al., 2000; Suzuki et al., 2002). Smad7 interaction leads to the ubiquitination and degradation of the receptors with the help of the E3 ubiquitin ligases, the Smad ubiquitination regulatory factors (Smurfs) (Ebisawa et al., 2001; Tajima et al., 2003). The TGF-β receptor-Smad7-Smurf complex is routed via caveolin-rich membrane structures and internalized via caveolin-positive vesicles toward the proteasome for degradation (Di Guglielmo et al., 2003) (Figure 1). Below, we discuss this process in the context of the role of Smad7 in the termination of TGF-β signaling.

Features of Smad Proteins
The first intracellular mediator of TGF-β signaling, MAD, was identified in *Drosophila* (Sekelsky et al., 1995), quickly followed by the discovery of orthologs in worm and vertebrates, which were named "Smad" (Derynck et al., 1996). Among the three classes of Smads, only R-Smads are directly phosphorylated and activated by the type I receptor kinases. Smad2 and Smad3 respond to signaling by the TGF-β subfamily and Smads 1, 5, and 8 primarily by the BMP subfamily (Figure 2).

The R-Smad and Co-Smad proteins, with around 500 amino acids in length, contain two conserved structural domains, the N-terminal MH1 domain and the C-terminal MH2 domain (Figure 6A). The R-Smads, but not the Co-Smads, contain a characteristic SXS motif at their C termini. The MH1 (MAD-homology 1) domain of Smad4
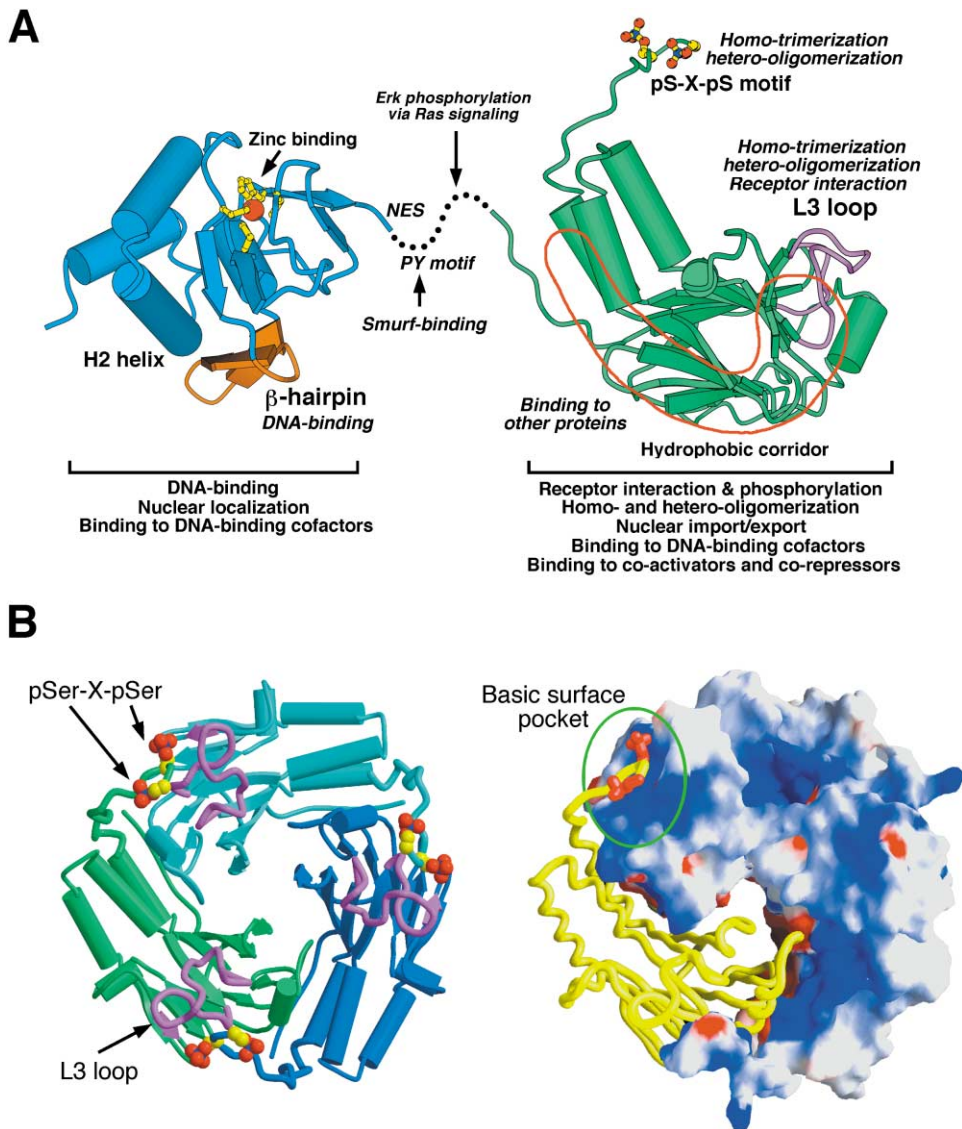
Figure 6. Structure and Function of the Smad Protein

(A) Shown here is a phosphorylated R-Smad. The MH1 and MH2 domains are colored cyan and green, respectively. The DNA binding hairpin is highlighted in orange. As predicted (Grishin, 2001), the MH1 domain also contains a tightly bound zinc atom as observed in a high-resolution crystal structure (Chai et al., 2003). The L3 loop of the MH2 domain is colored purple. The C-terminal pSer-X-pSer motif is shown in ball-and-stick representation.

(B) Phosphorylation of the SXS motif drives formation of homomeric as well as heteromeric complexes. Shown here is the homotrimeric structure of the Smad2 MH2 domain with its C-terminal SXS motif homogeneously phosphorylated (Wu et al., 2001b). In the left panel, the three Smad2 monomers are shown in blue, cyan, and green, with the L3 loop highlighted in pink. The right panel, with two Smad2 monomers in surface representation and one in yellow coil, emphasizes the highly positively charged surface pocket next to the L3 loop that serves as the binding site for the pSer-X-pSer motif. This basic surface pocket is conserved among all R-Smad as well as Smad4, suggesting a similar interface between R-Smad and Smad4. The basic and acidic surfaces are colored blue and red, respectively.

and most R-Smads (except the most common splice form of Smad2) exhibits sequence-specific DNA binding activity, may play a role in nuclear import, and negatively regulates the function of the MH2 domain. The N-terminal domain of I-Smads displays weak sequence homology to the MH1 domain of R-Smads but does not bind to DNA. In contrast, the MH2 domain is highly conversed among all Smad proteins and is responsible for receptor interaction, formation of homomeric as well as heteromeric Smad complexes, and directly con-

tacting the nuclear pore complex for nucleocytoplasmic shuttling. Importantly, the phosphorylation of the C-terminal two serine residues in the SXS motif of the MH2 domain drives the activation of the R-Smad (Figure 6B). Both the MH1 and MH2 domains interact with a large number of proteins in the nucleus, effecting transcription.

Although the linker sequences between the MH1 and MH2 domains are divergent among Smads, these regions contain multiple phosphorylation sites, which

allow specific crosstalks with other signaling pathways, and a PY motif, which mediates specific interaction with the Smurfs. Smurf1 and Smurf2 are HECT-domain-containing E3 ubiquitin ligases that target Smads as well as Smad-associated TGF-β receptors for degradation by the 26S proteasome (Figure 1). Smad dephosphorylation also plays a role in the termination of the TGF-β signal although the phosphatase(s) involved remains to be identified.

Smad Recognition by the Activated
Receptor Complex

How does the phosphorylated type I receptor exhibit a significantly enhanced binding affinity for the R-Smad? One clue came from a structural comparison of the MH2 domains of Smad2 and Smad4, which revealed the presence of a much more positively charged surface patch on Smad2 than that on Smad4, located next to the L3 loop (Wu et al., 2000). Sequence analysis indicates that this comparison holds true between all R-Smads and the Co-Smad, Smad4. Thus this basic patch on R-Smad was postulated to be the binding site for the phosphorylated GS region of the type I receptor (Wu et al., 2000) (Figure 6). Indeed, mutation of one of the invariant residues, His331, in the basic patch of Smad2 leads to a reduction in its affinity for, and phosphorylation by, TβRI (Huse et al., 2001).

Although the predicted interaction between the phosphorylated GS region of the type I receptor and the basic patch of an R-Smad enhances binding affinity, it does little to control the signaling specificity. The R-Smad C-terminal sequence to be phosphorylated also contributes little to the specificity of the receptor-Smad interaction. How, then, is a specific R-Smad chosen by the activated receptors? The answer lies in the L45 loop of the receptor kinase domain, which is located immediately adjacent to the GS region and specifies interactions with the R-Smads (Chen et al., 1998; Feng and Derynck, 1997) (Figure 5). The corresponding specificity determinant in the R-Smad primarily involves the L3 loop (Chen et al., 1998; Lo et al., 1998). Matching sets of Smad L3 loop and receptor L45 loop thus determine the receptor-Smad choices delineated in Figure 2. However, other sequence elements of R-Smads may also play a role in this interaction (Huse et al., 2001). A structure of an activated type I receptor bound to an R-Smad should reveal the detailed mechanism for this recognition.

Regulation of Smad Access to the Receptors

The recognition of R-Smads by the receptors may be facilitated by auxiliary proteins. For example, Smad2 and Smad3 can be specifically immobilized near the cell surface by the Smad anchor for receptor activation, or SARA (Tsukazaki et al., 1998), through interactions between a peptide sequence of SARA and an extended hydrophobic surface area on Smad2/Smad3 (Wu et al., 2000). SARA contains a phospholipid binding FYVE domain, which targets the molecule to the membrane of early endosomes (Tsukazaki et al., 1998). These interactions allow more efficient recruitment of Smad2 or Smad3 to the receptors for phosphorylation (Tsukazaki et al., 1998). At steady state, the bulk of SARA and SARA bound Smad2 are located in early endosomes.

Receptor-mediated phosphorylation of SARA bound Smad2 occurs at the plasma membrane but is more efficient in SARA-rich early endosomes to which the activated receptor complex is internalized via clathrin-coated pits (Di Guglielmo et al., 2003; Hayes et al., 2002; Lu et al., 2002) (Figure 1).

The activated TGF-β receptor complex therefore undergoes endocytosis via two distinct routes, for two different purposes: it is internalized via coated vesicles to early endosomes for signaling and via caveolae to caveolin-positive vesicles for degradation (Di Guglielmo et al., 2003). Another FYVE-containing protein, Hgs, was found to cooperate with SARA on Smad signaling (Miura et al., 2000). More recently, other adaptor proteins, including Disabled-2 (Hocevar et al., 2001), Axin (Furuhashi et al., 2001), and the ELF β-spectrin (Tang et al., 2003) have been reported to facilitate TGF-β signaling by linking Smad2/Smad3 to the receptor complex. However, the molecular mechanisms behind these interesting observations remain unclear.

Mechanism of Smad Phosphorylation
and Activation

R-Smads are directly phosphorylated by the activated type I receptors (Kretzschmar et al., 1997; Macias-Silva et al., 1996). The structure of a Smad MH2 domain comprises a central β sandwich, capped on one end by a three-helix bundle and on the other end by a collection of three surface loops and two auxiliary α helices (Shi, 2001) (Figure 6A). In the crystal structures of the MH2 domain from the unphosphorylated R-Smads, the C-terminal 10 residues, including the characteristic SSXS motif at the extreme C terminus, are completely flexible and disordered (Qin et al., 2001; Shi, 2001; Wu et al., 2000). Several lines of evidence demonstrated that phosphorylation of an R-Smad takes place in the C-terminal two serine residues within the flexible SSXS motif (Abdollah et al., 1997; Souchelnytskyi et al., 1997).

Phosphorylation destabilizes Smad interaction with SARA, allowing dissociation of Smad from the complex and the subsequent exposure of a nuclear import region on the Smad MH2 domain (Xu et al., 2000). In addition, R-Smad phosphorylation augments its affinity for Smad4. The association of these two proteins nucleates the assembly of transcriptional regulation complexes.

Using glutamate to simulate the phosphorylated serine residues, the MH2 domain of Smad1 and Smad3 was shown to exhibit a greater propensity for the formation of a homotrimer as well as a heteromeric Smad complex with Smad4 (Chacko et al., 2001). Mutational analysis revealed that the residues in the L3 loop region of one Smad3 monomer are required for binding to the pseudophosphorylated tail of an adjacent Smad3 monomer (Chacko et al., 2001).

Using a protein-phosphopeptide ligation approach, Smad2 and Smad3 proteins with the C-terminal two serine residues of their SXS motif homogeneously phosphorylated were generated (Wu et al., 2001b). Although the unphosphorylated Smad proteins exhibit a weak affinity for homotrimers, the phosphorylated Smad2 or Smad3 forms an extremely stable homotrimer. Structural analysis of the phosphorylated Smad2-MH2 reveals the molecular basis for the formation of this stable

homotrimer (Wu et al., 2001b) (Figure 6B). The C-terminal pSer-X-pSer motif of one Smad2 molecule is nestled in a positively charged surface pocket of the adjacent molecule through numerous specific hydrogen bonds. In addition to these phosphorylation-specific contacts, there is also a large protein-protein interface between adjacent Smad2 molecules, similar to that observed in the constitutively homotrimeric structure of Smad4 (Shi, 2001).

Importantly, the conformation of all structural elements in the MH2 domain of Smad2, except the N and C termini, remains unchanged before and after phosphorylation (Wu et al., 2000; Wu et al., 2001b). This experimental observation is in contrast to a postulated conformational change of Smad1 upon phosphorylation (Qin et al., 2001), derived from comparing the structure of an unphosphorylated Smad1 (Qin et al., 2001) with that of an unphosphorylated Smad2 (Wu et al., 2000). In addition, the observed conformational change at the N terminus of Smad2-MH2, involving a large movement of the B1′ strand, provides a plausible explanation to the decreased binding affinity for SARA (Wu et al., 2001b), as this β strand is also required for interaction with SARA (Wu et al., 2000).

pSer-X-pSer as the Defining Motif
of the TGF-β Pathway

The phosphorylated Smad2 structure not only explains the molecular mechanism for the formation of a homotrimer for R-Smad, but also reveals significant insights into the mechanism of R-Smad binding to Smad4. In the structure of the phosphorylated Smad2, the pSer-X-pSer motif is primarily coordinated by four important residues in the positively charged surface pocket; all four residue are preserved in Smad4, strongly indicating that a similar surface pocket on Smad4 serves as the binding site for the pSer-X-pSer motif of the R-Smads in the heteromeric Smad complex (Wu et al., 2001b). This conclusion is supported by biochemical and mutational analyses (Chacko et al., 2001; Wu et al., 2001b).

The stoichiometry of the heteromeric Smad complexes has been subjected to much debate (Chacko et al., 2001; Kawabata et al., 1998; Shi, 2001). Using isolated MH2 domains, strong evidence was presented either to favor a heterotrimer model (Chacko et al., 2001; Qin et al., 2001) or to support a heterodimer model between Smad4 and the R-Smad (Wu et al., 2001a; Wu et al., 2001b). Investigation of the Smad stoichiometry in cells suggests a complex situation (Jayaraman and Massagué, 2000). More recently, it was suggested that, depending on the gene promoter context, both a heterotrimer and a heterodimer are possible (Inman and Hill, 2002). Indeed, this issue can only be addressed in a biologically relevant manner by investigating the active heteromeric Smad complexes in the nucleus.

Regardless of the stoichiometry for the heteromeric Smad complex, it has become clear that the interface between the C-terminal pSer-X-pSer motif of the R-Smad and the basic surface pocket of the Smad4 MH2 domain play a dominant role in the formation of a heteromeric complex (Wu et al., 2001b). This conclusion is supported by all published observations as well as the skewed distribution of the tumor-derived missense mutations.

The type I receptor interaction with R-Smad is also likely mediated by the two contiguous pThr/Ser-X-pSer motifs of the phosphorylated GS domain and the basic surface pocket of the MH2 domain (Figure 5). Therefore, the pSer-X-pSer interaction with the MH2 basic surface pocket represents the signature interaction of the receptor serine/threonine kinase signaling pathway.

Mechanism of Smad Nucleocytoplasmic Shuttling

Receptor-mediated phosphorylation of the R-Smads induces their accumulation in the nucleus. Since the activated type I receptors form a relatively stable complex with the R-Smads, how do these R-Smad proteins dissociate from the receptor after phosphorylation? The answer resides, at least in part, in the ability of the phosphorylated SXS motif to compete with the GS region of the receptor for binding to the basic surface pocket located next to the L3 loop of the R-Smads (Wu et al., 2001b) (Figure 5). This model predicts that loss of phosphorylation on the SXS motif may lead to prolonged association between an R-Smad and the receptor kinases, a prediction well supported by the evidence (Abdollah et al., 1997; Lo et al., 1998; Souchelnytskyi et al., 1997). Additionally, phosphorylation of Smad2 decreases its affinity for the binding site of SARA (Xu et al., 2000).

In the basal state, R-Smads are predominantly localized in the cytoplasm, whereas the I-Smads tend to be nuclear. Smad4 is distributed in both the cytoplasm and the nucleus. After receptor activation, the phosphorylated R-Smads are translocated into the nucleus. The Smad MH1 domain contains a conserved lysine-rich helix—the helix H2—located next to the DNA binding motif β-hairpin (Figure 6A) (Chai et al., 2003; Shi et al., 1998). The solvent-exposed C-terminal portion of this helix (KKLKK), invariant among all R-Smads, has been suggested to act as a nuclear localization signal (NLS) for Smad1 and Smad3 (Xiao et al., 2000a, 2001). This activity has been shown to depend on Smad phosphorylation (Kurisaki et al., 2001; Xiao et al., 2000b). This protein interaction is different from the interaction of the classical importin pathway. In that pathway, importin β binds to importin α, which recognizes as an NLS an extended lysine-rich sequence in the cargo molecule (Gorlich and Kutay, 1999; Mattaj and Englmeier, 1998). In the Smad MH1 nuclear import studies, however, the basic sequence in question is part of an α-helix (Chai et al., 2003; Shi et al., 1998), and it binds to importin β, but not importin α (Kurisaki et al., 2001; Xiao et al., 2000b).

An alternative mechanism for the nuclear import and export of Smad2 (and Smad3) has been elucidated based on the observation that the MH2 domain binds directly to components of the nuclear pore complex, the nucleoporins CAN/Nup214 and Nup153 (Xu et al., 2002). Binding is mediated by the FG (Phe-Gly) repeat region of these nucleoporins. This allows Smad import into the nucleus in the absence of added importins and export independently of the general export factor Crm-1 (Xu et al., 2002). By directly contacting the nuclear pore complex, Smad2 undergoes constant shuttling, providing a dynamic pool that is competitively drawn by cyto-
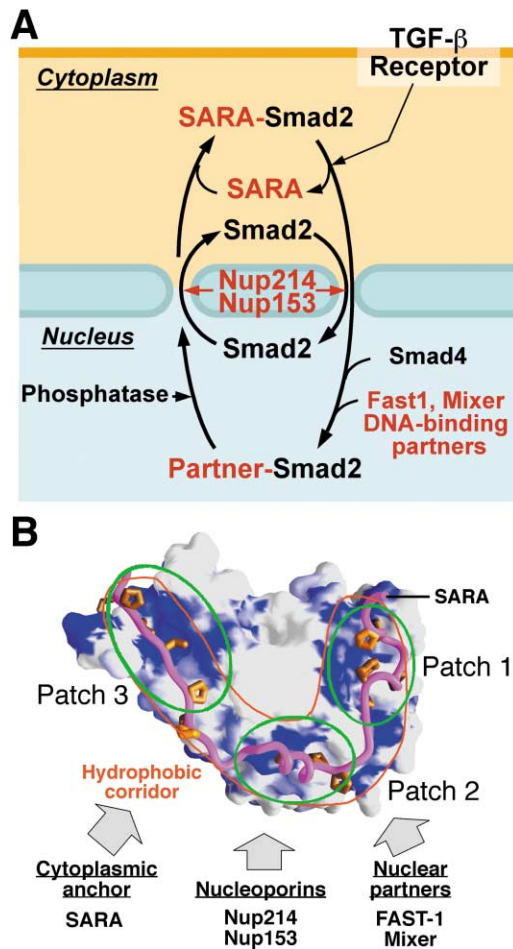
Figure 7. Nucleocytoplasmic Shuttling of the Smad Proteins

(A) A schematic diagram showing the nucleocytoplasmic shuttling of Smad2, with proteins that compete for Smad2 binding shown in red.

(B) A hydrophobic corridor on R-Smads is the common interaction site for many Smad cofactors. The hydrophobic surface patch of Smad2 (Wu et al., 2000) is colored blue. The bound SARA fragment is shown in pink.

plasmic and nuclear signal transduction partners (Figures 1 and 7A).

The nucleoporin-interacting site in Smad2 overlaps the SARA binding site in the MH2 domain as defined by the crystal structure of a Smad2-SARA complex (Wu et al., 2000) (Figure 7B). This site, referred to as the "hydrophobic corridor," also serves as the common binding site for SARA and nuclear transcription factors as they share a conserved Smad interacting motif or SIM (Randall et al., 2002). SARA in the cytoplasm and Smad partners in the nucleus compete with CAN/Nup214 and Nup153 for binding to the hydrophobic corridor (Xu et al., 2002) (Figure 7B). Receptor-mediated phosphorylation of Smad2 decreases its binding affinity for SARA, but not for CAN/Nup214 or Nup153 (Tsukazaki et al., 1998; Xu et al., 2000, 2002).

Continuous nucleocytoplasmic shuttling of the Smad proteins appears to be a key event in TGF-β signaling (Inman et al., 2002; Xu et al., 2002) (Figure 7A). Following

TGF-β stimulation of epithelial cells, receptors remain active for a few hours, and this activity is required to maintain the active Smads in the nucleus for TGF-β-regulated transcription (Inman et al., 2002). Continuous shuttling of R-Smad with repeated cycles of receptor-mediated phosphorylation, and dephosphorylation, permits constant sensing on the activation status of the TGF-β receptor and ensures an efficient termination of signaling upon receptor inactivation.

Smad4 accumulates in the nucleus by association with activated R-Smads (Hoodless et al., 1999; Liu et al., 1997; Watanabe et al., 2000). However, Smad4 also undergoes continuous nucleocytoplasmic shuttling on its own, independently of TGF-β signaling (Pierreux et al., 2000; Watanabe et al., 2000). The nuclear import signal in Smad4 includes not only just lysine residues in the H2 helix of the MH1 domain, but also a basic residue Arg81 in the β hairpin (Xiao et al., 2003). A nuclear export signal is present in the linker region of Smad4 and may be masked through the formation of a heteromeric complex with R-Smads (Inman et al., 2002; Watanabe et al., 2000; Xiao et al., 2001). An alternatively spliced form of Smad4 in mammals (Pierreux et al., 2000) and a separate gene product—Smad4β, also known as Smad10—in Xenopus (Watanabe et al., 2000) lack the NES and are constitutively nuclear.

Negative Regulation of Smad Nuclear Accumulation
Ras and TGF-β signals act cooperatively as well as antagonistically during development and in oncogenesis. Although TGF-β can override the proliferative effects of EGF and other Ras-activating mitogens in normal epithelial cells, oncogenic activation of Ras suppresses the cytostatic effects of TGF-β. As Ras has diverse effectors and targets in the cell, its interplay with TGF-β signaling is likely to occur at multiple levels, many of which involve indirect molecular interaction. However, it has been shown that Ras-mediated activations of Erk MAP kinases results in the phosphorylation of Smads 1, 2, and 3 at MAP kinase sites in the linker sequence between the MH1 and MH2 domains, and this attenuates agonist-induced nuclear accumulation of these Smads and alters Smad-dependent transcription (Kretzschmar et al., 1999, 1997). Oncogenic Ras also appeared to induce degradation of Smad4 (Saha et al., 2001).

The effect of these MAP kinase phosphorylation sites on Smad nuclear accumulation is subtle in some cell types and conditions but profound in others. For example, in Xenopus frog embryos, Activin signaling leads to Smad2 nuclear accumulation and, with that, induction of mesodermal marker genes for many hours; this ends abruptly with a nuclear exclusion of Smad2 dependent on the MAP kinase phosphorylation sites in the linker region (Grimm and Gurdon, 2002). The mechanism mediating Smad nuclear exclusion via MAP kinase phosphorylation is not known. It could involve direct effects on the Smad nuclear import or export processes or, alternatively, effects on Smad interaction with functional partner proteins that secondarily affect the subcellular distribution of Smads. On the other hand, the JNK pathway has also been shown to have positive effects on Smad function, although the phosphorylation sites involved in this process remain under investigation (Brown et al., 1999b).
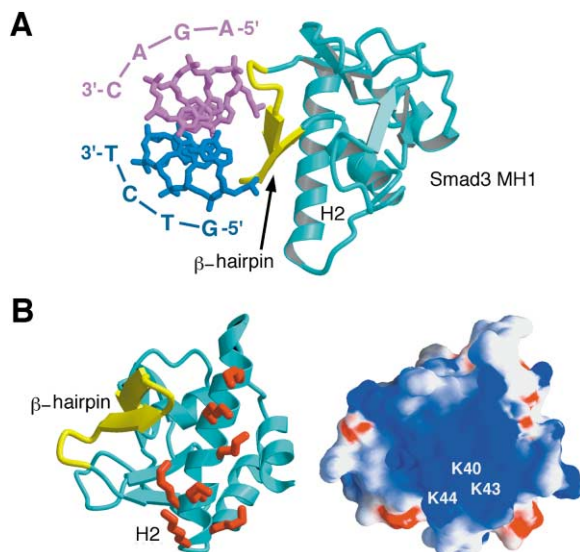
Figure 8. Specific DNA Recognition by the Smad Proteins

(A) Structure of a Smad3 MH1 domain bound to SBE (Chai et al., 2003; Shi et al., 1998). The DNA binding β-hairpin is highlighted in yellow. The two DNA strands are colored blue and magenta, respectively.

(B) Closeup views of the highly positively charged helix H2. The left panel shows the ribbon diagram of Smad3 MH1 structure, with seven Lys residues shown in red. The positions of the H2 helix and the β-hairpin are indicated. The right panel shows the surface electrostatic potential of Smad3 MH1 domain.

Similar to the effect of Erk, calcium-calmodulin-dependent protein kinase II, acting downstream of other growth factor receptors, inhibits TGF-β signaling through phosphorylation of Smad2 at several residues in the linker region (Wicks et al., 2000). Although these residues are different from the MAP kinase sites, their phosphorylation also inhibited Smad2 nuclear accumulation (Wicks et al., 2000).

Basis for DNA Recognition by Smad Proteins
Smad4 and all R-Smads except Smad2 bind to DNA in a sequence-specific manner. The minimal Smad binding element (SBE), initially identified as the optimal DNA binding sequence for Smad3 and Smad4, contains only four base pairs, 5′-AGAC-3′ (Dennler et al., 1998; Yingling et al., 1997; Zawel et al., 1998), although most naturally occurring DNA sequences contains an extra base C at the 5′ end. The crystal structure of the Smad3 MH1 domain bound to SBE revealed that a highly conserved β hairpin makes specific contacts to three bases of the SBE (Chai et al., 2003; Shi et al., 1998) (Figure 8A). The conserved nature of the DNA binding β hairpin as well as its surrounding sequence elements among all R-Smads strongly suggests that other R-Smads should specifically bind to SBE as well (Shi et al., 1998). Compared to other Smads, the most common splice form of Smad2 contains a unique 30 residue insertion between the DNA binding β hairpin and the helix H2; this sequence variation resulted in the poor DNA binding ability of Smad2, presumably due to disruption of the conformation of the DNA binding hairpin (Shi et al., 1998). In support of this explanation, DNA binding was restored in the alterna-

tively spliced variant of Smad2 lacking this insertion (Yagi et al., 1999).

The relatively low specificity of DNA binding by Smads has created difficulty in the identification of biologically relevant Smad-responsive DNA elements. For example, while Smad4, Smad3, and Smad1 bind specifically to SBE (Johnson et al., 1999; Shi et al., 1998; Zawel et al., 1998), they have also been reported to bind to G/C-rich sequences (Ishida et al., 2000; Kim et al., 1997; Labbe et al., 1998). This challenge is further confounded by the highly positively charged helix H2 of the MH1 domain, which resides next to the DNA binding β-hairpin in the crystal structure (Shi, 2001) (Figure 8B). It has been documented that clusters of lysine residues generally prefer G/C-rich sequences (Choo and Klug, 1997). Can the Smad MH1 domain interact with two distinct sets of DNA sequences using two different sequence motifs? If the answer were positive, this would represent the first such case.

Mechanism of Target Gene Selection
Cell stimulation with TGF-β leads immediately to positive and negative changes in the expression of several hundred genes (Kang et al., 2003). Both activation and repression of gene expression use the same set of activated Smad proteins. Further compounding this complexity, many of these gene responses depend on the cell type and other conditions affecting the cell at the time of TGF-β stimulation.

A general hypothesis for how cells read TGF-β signals posits that Smad access to target genes and the recruitment of transcriptional coactivators or corepressors to such genes depend on cell-type specific partner proteins (Massagué, 2000). This hypothesis appears to be true even for *Smad7*, whose promoter contains two copies of SBE and whose expression is activated by both TGF-β signaling and BMP signaling largely in a cell type-independent manner. Binding to this promoter sequence involves Smad3, Smad4, and at least one additional component (Denissova et al., 2000; Inman and Hill, 2002).

Most Smad-responsive enhancers, however, contain only one copy of the SBE. Because of the relatively low specificity of DNA binding by individual Smad proteins, Smads must cooperate among each other and with other DNA binding proteins to elicit specific transcriptional responses (Figure 9A). Some of these factors may be ubiquitous and mediate the same response in all cell types (Figure 9A, *gene 1*). Others Smad partners or their activators may be cell-type restricted, giving rise to cell-type dependent gene responses (Figure 9A, *genes 2–4*). Remarkably, members from many different families of DNA binding proteins—forkhead (e.g., Fast1), homeobox (e.g., Mixer), E-box, Jun/Fos, Runx, CREBP, and E2F—have been shown to function as Smad partners in this fashion. Since this topic has been extensively reviewed recently (Attisano and Wrana, 2000; Massagué and Wotton, 2000; ten Dijke et al., 2000), we will restrict our discussion to recent progress and focus on the shared mechanisms.

Mechanisms of Transcriptional Control
The MH2 domain of the Smad proteins exhibits trans-activation function as a heterologous fusion with GAL4
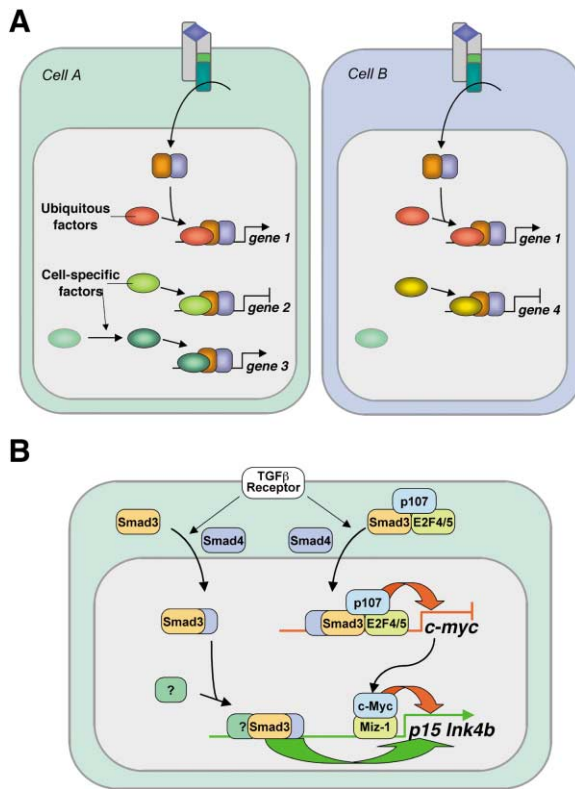
Figure 9. Transcriptional regulation by the Smad proteins

(A) Context-dependent transcriptional responses by the Smad complexes. The ubiquitous factors may give rise to a uniform response in different cell types. The cell-specific factors dictate differential responses in different cell types for the same Smad proteins.
(B) A schematic diagram of the transcriptional regulation on the *Myc* and *p15Ink4b* genes by the Smad proteins in epithelial cells.

DNA binding domain, suggesting that Smads may directly associate with the basal transcriptional machinery. Indeed, the MH2 domain of both R-Smads and Smad4 interacts with the transcriptional coactivators CBP, p300, ARC105, and Smif (Bai et al., 2002; Derynck et al., 1998; Kato et al., 2002; Massagué and Wotton, 2000). How, then, does TGF-β signaling via Smad proteins lead to activation or repression in the same cell at the same time, depending on the target gene? Analysis of the cell cycle arrest response to TGF-β in epithelial cells, which has been intensely pursued because of its relevance to cancer, has provided insights into this question.

Two of the most important cytostatic gene responses to TGF-β in epithelial cells are activation of *p15Ink4b*, which encodes a cyclin-dependent kinase (CDK) inhibitor, and repression of c-*myc*, which encodes a potent transcriptional activator of genes required for growth and proliferation (Massagué et al., 2000). A TGF-β-activated Smad complex that may also include Sp1 and other as yet unidentified factors recognizes the *p15Ink4b* promoter for activation (Figure 9B) (Feng et al., 2000; Seoane et al., 2001). How in the same cells the activated Smad proteins mediate repression of c-*myc* was recently characterized (Chen et al., 2002). A complex containing Smad3, the transcription factors

E2F4/5 and DP1, and the corepressor p107 preexists in the cytoplasm. In response to TGF-β, this complex moves into the nucleus and associates with Smad4, recognizing a composite Smad-E2F site on c-*myc* for repression (Figure 9B). Previously known as the ultimate recipients of CDK regulatory signals, E2F4/5 and p107 act here as transducers of TGF-β receptor signals upstream of CDK. Since Myc directly represses the expression of *p15Ink4b* through association with the transcription factor Miz-1 (Seoane et al., 2001; Staller et al., 2001), the Smad-E2F-p107-mediated repression of c-*myc* also relieves Myc-mediated repression of *p15Ink4b* (Figure 9B). Thus, the Smad proteins can mediate transcriptional activation or repression depending on their associated partners.

Regulation of Smad-Dependent Transcription
Among the negative regulators of Smad transcriptional function, c-Ski and SnoN are two highly conserved members of the Ski family of protooncoproteins. Ski or SnoN antagonize TGF-β signaling through direct interactions with Smad4 and the R-Smads (Liu et al., 2001; Wang et al., 2000). The mechanism of Ski-mediated repression of TGF-β signaling was primarily attributed to transcriptional modulation, through recruitment of the nuclear transcriptional corepressor (N-CoR) and histone deacetylase (HDAC) as well as interference of Smad-mediated binding to the transcriptional coactivator, p300/CBP (Liu et al., 2001). One additional mechanism was provided by the recent structure of the Smad4 binding domain of c-Ski in complex with the MH2 domain of Smad4, which reveals an overlap between the Ski binding and R-Smad binding surfaces on Smad4 (Wu et al., 2002). Indeed, Ski competes with R-Smads for interaction with Smad4 and disrupts the formation of a functional complex between Smad4 and R-Smads. Because Ski contains separate sequence elements for interaction with Smad4 and R-Smads, both Smad4 and R-Smad may remain attached to Ski after disruption of the functional complex (Luo et al., 1999; Stroschein et al., 1999; Wu et al., 2002).

SnoN-mediated negative regulation on the Smad proteins is removed during TGF-β signaling by at least two distinct ways. In the presence of TGF-β signaling, Smad2 interacts with both SnoN and Smurf2, allowing the HECT domain of Smurf2 to target SnoN for ubiquitin-mediated degradation by the proteasome (Bonni et al., 2001). Smad2 and Smad3 can also recruit the E3 ubiquitin ligase anaphase promoting complex (APC), resulting in the ubiquitination and degradation of Smad bound SnoN (Stroschein et al., 2001; Wan et al., 2001).

Smads can also bind to transcriptional corepressor TGIF (Wotton et al., 1999a), which in turn recruits HDAC and other repressors via the adaptor protein Sin3 and CtBP to repress transcription (Wotton et al., 2001, 1999a, 1999b). Like Ski and SnoN, TGIF is thought to act as an inhibitor of TGF-β-dependent gene activation rather than as a mediator of TGF-β-dependent gene repression.

TGIF additionally provides a point for negative regulation of TGF-β signaling by the Ras-MEK-MAPK pathway. Ras-activating growth factors including EGF and HGF, as well as Ras-activating oncogenic mutations, signal-
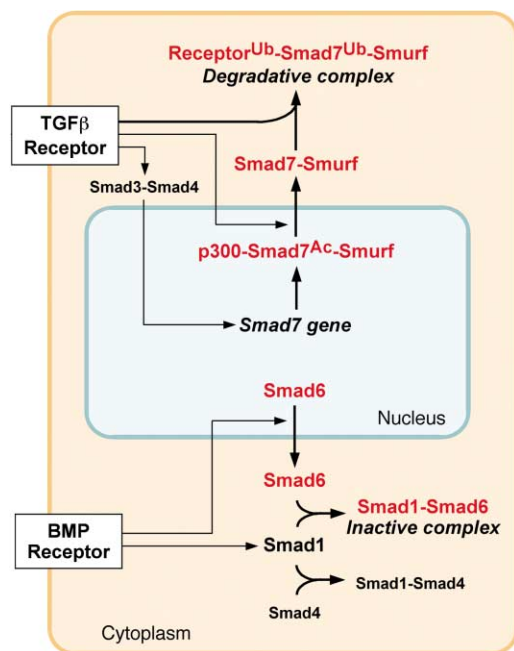
Figure 10. A Schematic Diagram on the Function of the Inhibitory Smads (I-Smads)

Transcriptionally activated by TGF-β signaling, Smad7 promotes the ubiquitination and degradation of the receptors via Smurf. Smad6 competes with Smad1 for binding to the Co-Smad, Smad4.

ing via MEK, cause the phosphorylation of TGIF at two Erk MAP kinase sites. This leads to stabilization of the TGIF protein, favoring the formation of Smad2-TGIF corepressor complexes in response to TGF-β (Lo et al., 2001). Thus, the Smad and Ras pathways can intersect at both the Smad nuclear translocation level and the transcriptional level in the nucleus.

Ending Smad Signaling
Dephosphorylation by as yet unidentified phosphatases (Randall et al., 2002) as well as ubiquitination and proteasome-mediated degradation of the activated R-Smads are two mechanisms for the termination of Smad signaling (Figure 1). Smurf1 targets Smad1 and Smad5 for destruction in the cytoplasm of unstimulated cells (Zhu et al., 1999). This function may be important for the maintenance of the basal state in the unstimulated cells. However, activated Smad2 is ubiquitinated in the nucleus and undergoes proteasome-mediated degradation (Lo and Massagué, 1999); this process may involve Smurf2, which has extensive sequence similarity to Smurf1 (Lin et al., 2000; Zhang et al., 2001). Ubiquitination of activated Smad3 appears to be mediated by a different E3, the SCF/Roc1 complex (Fukuchi et al., 2001).

Smurf1 and 2 also mediate ubiquitination of activated TGF-β receptors, leading to their degradation in the proteasome (Ebisawa et al., 2001; Tajima et al., 2003). The inhibitory Smad, Smad7, plays an important role in this process. Smad7 resides in the nucleus in the basal state and moves to the plasma membrane upon TGF-β or BMP stimulation (Itoh et al., 1998) (Figure 10). Both

Smurf1 and Smurf2 form a stable complex with Smad7. Using their N-terminal C2 domain, Smurf1 and Smurf2 target the Smurf-Smad7 complex to the plasma membrane, where Smad7 directly binds to the activated type I TGF-β receptor and inhibits phosphorylation of the R-Smads (Kavsak et al., 2000; Suzuki et al., 2002). In the membrane complex, Smurf1 also directly mediates the ubiquitination and turnover of the receptors (Ebisawa et al., 2001; Tajima et al., 2003). Smad7 itself undergoes ubiquitination and degradation in this process. However, this negative feedback loop is perpetuated by the ability of TGF-β (and BMPs) to transcriptionally activate *Smad7*, thus ensuring a steady supply of the protein as it undergoes degradation (Figure 10).

In the nucleus, Smad7 is associated with the nuclear acetyl transferase p300, which acetylates lysine residues 64 and 70 in Smad7, protecting it against Smurf-mediated ubiquitination of the same residues (Gronroos et al., 2002). Smad7 acetylation is lost as the protein leaves the nucleus in response to TGF-β stimulation and separates from p300. Although R- and Co-Smads also associate with p300 and the related acetyl transferase CBP in the context of assembling transcriptional complexes, no evidence has been found that R- or Co-Smads undergo acetylation.

Smad6 acts in a different manner. It competes with the activated Smad1 for binding to Smad4, serving as an inhibitor of BMP signaling (Hata et al., 1998). Smad6 can be sequestered by the protein AMSH upon nuclear export in response to BMP (Itoh et al., 2001). The WD40 domain-containing protein STRAP is thought to facilitate Smad7 recruitment to the activated TGF-β receptor complex (Datta and Moses, 2000). Beyond this, the elements responsible for the constitutive nuclear localization of I-Smads or their signaling-dependent export remain unknown.

These observations underscore the complex mechanisms controlling TGF-β signaling and suggest the involvement of additional factors that may regulate the stability of Smad proteins at both the basal and the activated states. Furthermore, Smad7 is used not only to establish a negative feedback loop in TGF-β signaling, but also to negatively regulate this pathway by antagonistic signals. Thus, the proinflammatory cytokines interferon-γ and tumor necrosis factor-α, signaling via Stat1 and NF-κB, respectively, activate *Smad7* expression with an attendant inhibition of TGF-β signaling (Bitzer et al., 2000; Ulloa et al., 1999).

Perspectives
In essence, the TGF-β signal transduction pathway as we know it involves a ligand-activated receptor complex that promotes the formation of Smad transcriptional complexes. The simplicity of this pathway is in sharp contrast with the great diversity of cell-specific gene responses that it triggers and its dependence on the context. In fact, by focusing here on the central components of the TGF-β pathway, we have largely sidestepped two of its main defining features: the cell-type dependence of its actions and its many links with other signaling pathways (reviewed in Massagué et al., 2000). Much work is needed for the delineation of cell-type specific gene responses that mediate the action of the

TGF-β family in tissue development and maintenance and the pathological manifestations of these processes. The general concepts and specific examples of TGF-β gene responses reviewed here provide only a general framework for future work.

Likewise, it is understood that signaling pathways are embedded in the overall signaling network of the cell. Therefore, the signal flow through the TGF-β pathway is constantly being enhanced, suppressed or modified by other signals imping ing on the cell. Direct biochemical effects of Ras, Stat, or NF-κB pathways on Smads or some of their immediate regulators have been described. However, many more signals are likely to influence the cellular response to TGF-β by affecting the activity of Smad transcriptional partners and, in so doing, the composition of the Smad transcriptional complexes that dictate the cell-type specific response to TGF-β. Most of the Smad partners identified to date (e.g., Fast1, Mixer, Jun/Fos, Runx, ATF3, E2F4/5) are indeed highly responsive to different inputs. Thus, the final step in Smad signaling provides a fertile ground for the delineation of cell-type specific responses to TGF-β signals.

Finally, despite much progress over the past decade, many unanswered questions remain about the core components of the TGF-β signal pathway themselves. For example, a better understanding of the structural basis for the assembly of a TGF-β receptor complex on the cell surface or a Smad transcriptional complex on the DNA is needed. The possible existence of noncanonical forms of TGF-β signaling also deserves attention: signaling via type II receptors without the type I, via type I receptors without Smads, or via R-Smads without Smad4, remain as open questions. The observed ability of TGF-β to activate Rho GTPases (Bhowmick et al., 2001), protein phosphatase 2A (Petritsch et al., 2000), and MAP kinases (Yu et al., 2002; Itoh et al., 2003) by Smad-independent mechanisms offer intriguing possibilities for future research. Another decade may have to elapse before a satisfactory level of understanding about TGF-β signaling is achieved.

References

Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J.L. (1997). TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J. Biol. Chem. 272, 27678–27685.

Abreu, J.G., Ketpura, N.I., Reversade, B., and De Robertis, E.M. (2002). Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-β. Nat. Cell Biol. 4, 599–604.

Attisano, L., and Wrana, J.L. (2000). Smads as transcriptional comodulators. Curr. Opin. Cell Biol. 12, 235–243.

Bai, R.Y., Koester, C., Ouyang, T., Hahn, S.A., Hammerschmidt, M., Peschel, C., and Duyster, J. (2002). SMIF, a Smad4-interacting protein that functions as a co-activator in TGFβ signalling. Nat. Cell Biol. 4, 181–190.

Balemans, W., and Van Hul, W. (2002). Extracellular regulation of

BMP signaling in vertebrates: a cocktail of modulators. Dev. Biol. 250, 231–250.

Bhowmick, N.A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C.A., Engel, M.E., Arteaga, C.L., and Moses, H.L. (2001). Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell 12, 27–36.

Bitzer, M., von Gersdorff, G., Liang, D., Dominguez-Rosales, A., Beg, A.A., Rojkind, M., and Bottinger, E.P. (2000). A mechanism of suppression of TGF-β/SMAD signaling by NF-kappa B/RelA. Genes Dev. 14, 187–197.

Boesen, C.C., Radaev, S., Motyka, S.A., Patamawenu, A., and Sun, P.D. (2002). The 1.1 Å crystal structure of human TGF-β type II receptor ligand binding domain. Structure 10, 913–919.

Bonni, S., Wang, H.R., Causing, C.G., Kavsak, P., Stroschein, S.L., Luo, K., and Wrana, J.L. (2001). TGF-β induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. Nat. Cell Biol. 3, 587–595.

Brown, C.B., Boyer, A.S., Runyan, R.B., and Barnett, J.V. (1999a). Requirement of type III TGF-β receptor for endocardial cell transformation in the heart. Science 283, 2080–2082.

Brown, J.D., DiChiara, M.R., Anderson, K.R., Gimbrone, M.A., Jr., and Topper, J.N. (1999b). MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. J. Biol. Chem. 274, 8797–8805.

Brunet, L.J., McMahon, J.A., McMahon, A.P., and Harland, R.M. (1998). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280, 1455–1457.

Chacko, B.M., Qin, B., Correia, J.J., Lam, S.S., de Caestecker, M.P., and Lin, K. (2001). The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. Nat. Struct. Biol. 8, 248–253.

Chai, J., Wu, J.-W., Massagué, J., Pavletich, N.P., and Shi, Y. (2003). Features of a Smad3 MH1-DNA complex: roles of water and zinc in DNA binding at 2.4 Å resolution. J. Biol. Chem., in press.

Chen, C.R., Kang, Y., Siegel, P.M., and Massagué, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-myc repression. Cell 110, 19–32.

Chen, Y.-G., Hata, A., Lo, R.S., Wotton, D., Shi, Y., Pavletich, N., and Massagué, J. (1998). Determinants of specificity in the TGF-β signal transduction. Genes Dev. 12, 2144–2152.

Cheng, S.K., Olale, F., Bennett, J.T., Brivanlou, A.H., and Schier, A.F. (2003). EGF-CFC proteins are essential coreceptors for the TGF-β signals Vg1 and GDF1. Genes Dev. 17, 31–36.

Choo, Y., and Klug, A. (1997). Physical basis of a protein-DNA recognition code. Curr. Opin. Struct. Biol. 7, 117–125.

Datta, P.K., and Moses, H.L. (2000). STRAP and Smad7 synergize in the inhibition of transforming growth factor β signaling. Mol. Cell. Biol. 20, 3157–3167.

De Caestecker, M., and Meyrick, B. (2001). Bone morphogenetic proteins, genetics and the pathophysiology of primary pulmonary hypertension. Respir. Res. 2, 193–197.

Denissova, N.G., Pouponnot, C., Long, J., He, D., and Liu, F. (2000). Transforming growth factor β-inducible independent binding of SMAD to the Smad7 promoter. Proc. Natl. Acad. Sci. USA 97, 6397–6402.

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J.-M. (1998). Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J. 17, 3091–3100.

Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.-H., Kern, S.E., Massagué, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., et al. (1996). Nomenclature: vertebrate mediators of TGFβ family signals. Cell 87, 173.

Derynck, R., Zhang, Y., and Feng, X.H. (1998). Smads: transcriptional activators of TGF-β responses. Cell 95, 737–740.

Di Guglielmo, G.M., Le Roy, C., Davidson, A.F., and Wrana, J.L. (2003). Distinct endocytic pathways regulate TGFβ receptor signaling and turnover. Nat. Cell Biol. 5, 410–421.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J. Biol. Chem. 276, 12477–12480.

Feng, X.-H., and Derynck, R. (1997). A kinase subdomain of transforming growth factor-β (TGF-β) type I receptor determines the TGF-β intracellular signaling specificity. EMBO J. 16, 3912–3923.

Feng, X.H., Lin, X., and Derynck, R. (2000). Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-β. EMBO J. 19, 5178–5193.

Fukuchi, M., Imamura, T., Chiba, T., Ebisawa, T., Kawabata, M., Tanaka, K., and Miyazono, K. (2001). Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol. Biol. Cell 12, 1431–1443.

Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K., and Kato, M. (2001). Axin facilitates Smad3 activation in the transforming growth factor β signaling pathway. Mol. Cell. Biol. 21, 5132–5141.

Gong, Y., Krakow, D., Marcelino, J., Wilkin, D., Chitayat, D., Babul-Hirji, R., Hudgins, L., Cremers, C.W., Cremers, F.P., Brunner, H.G., et al. (1999). Heterozygous mutations in the gene encoding noggin affect human joint morphogenesis. Nat. Genet. 21, 302–304.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. 15, 607–660.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. EMBO J. 21, 1743–1753.

Gray, P.C., Greenwald, J., Blount, A.L., Kunitake, K.S., Donaldson, C.J., Choe, S., and Vale, W. (2000). Identification of a binding site on the type II activin receptor for activin and inhibin. J. Biol. Chem. 275, 3206–3212.

Greenwald, J., Fischer, W.H., Vale, W.W., and Choe, S. (1999). Three-finger toxin fold for the extracellular ligand-binding domain of the type II activin receptor serine kinase. Nat. Struct. Biol. 6, 18–22.

Greenwald, J., Groppe, P., Gray, P., Wiater, E., Kwiatkowski, W., Vale, W.W., and Choe, S. (2003). The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. Mol. Cell 11, 605–617.

Grimm, O.H., and Gurdon, J.B. (2002). Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. Nat. Cell Biol. 4, 519–522.

Grishin, N.V. (2001). MH1 domain of Smad is a degraded homing endonuclease. J. Mol. Biol. 307, 31–37.

Gronroos, E., Hellman, U., Heldin, C.H., and Ericsson, J. (2002). Control of Smad7 stability by competition between acetylation and ubiquitination. Mol. Cell 10, 483–493.

Groppe, J., Greenwald, J., Wiater, E., Rodriguez-Leon, J., Economides, A.N., Kwiatkowski, W., Affolter, M., Vale, W.W., Belmonte, J.C., and Choe, S. (2002). Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. Nature 420, 636–642.

Harland, R.M. (2001). Developmental biology. A twist on embryonic signalling. Nature 410, 423–424.

Hart, P.J., Deep, S., Taylor, A.B., Shu, Z., Hinck, C.S., and Hinck, A.P. (2002). Crystal structure of the human TβR2 ectodomain-TGF-β3 complex. Nat. Struct. Biol. 9, 203–208.

Hata, A., Lagna, G., Massagué, J., and Hemmati-Brivalou, A. (1998). Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumour suppressor. Genes Dev. 12, 186–197.

Hayes, S., Chawla, A., and Corvera, S. (2002). TGF-β receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. J. Cell Biol. 158, 1239–1249.

Hocevar, B.A., Smine, A., Xu, X.X., and Howe, P.H. (2001). The adaptor molecule Disabled-2 links the transforming growth factor β receptors to the Smad pathway. EMBO J. 20, 2789–2801.

Hoodless, P.A., Tsukazaki, T., Nishimatsu, S., Attisano, L., Wrana, J.L., and Thomsen, G.H. (1999). Dominant-negative Smad2 mutants inhibit activin/Vg1 signaling and disrupt axis formation in Xenopus. Dev. Biol. 207, 364–379.

Huse, M., Chen, Y.-G., Massagué, J., and Kuriyan, J. (1999). Crystal structure of the cytoplasmic domain of the type I TGF-β receptor in complex with FKBP12. Cell 96, 425–436.

Huse, M., Muir, T.W., Xu, L., Chen, Y.-G., Kuriyan, J., and Massagué, J. (2001). The TGF-β receptor activation process: an inhibitor- to substrate-binding switch. Mol. Cell 8, 671–682.

Inman, G.J., and Hill, C.S. (2002). Stoichiometry of active Smad-transcription factor complexes on DNA. J. Biol. Chem. 277, 51008–51016.

Inman, G.J., Nicolas, F.J., and Hill, C.S. (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-β receptor activity. Mol. Cell 10, 283–294.

Ishida, W., Hamamoto, T., Kusanagi, K., Yagi, K., Kawabata, M., Takehara, K., Sampath, T.K., Kato, M., and Miyazono, K. (2000). Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. J. Biol. Chem. 275, 6075–6079.

Itoh, F., Asao, H., Sugamura, K., Heldin, C.-H., ten Dijke, P., and Itoh, S. (2001). Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. EMBO J. 20, 4132–4142.

Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C.-H., Heldin, N.-E., and ten Dijke, P. (1998). Transforming growth factor β1 induces nuclear export of inhibitory Smad7. J. Biol. Chem. 273, 29195–29201.

Itoh, S., Thorikay, M., Kowanetz, M., Moustakas, A., Itoh, F., Heldin, C.H., and ten Dijke, P. (2003). Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. J. Biol. Chem. 278, 3751–3761.

Jayaraman, L., and Massagué, J. (2000). Distinct oligomeric states of SMAD proteins in the TGF-β pathway. J. Biol. Chem. 275, 40710–40717.

Johnson, K., Kirkpatrick, H., Comer, A., Hoffmann, F.M., and Laughon, A. (1999). Interaction of Smad complexes with tripartite DNA-binding sites. J. Biol. Chem. 274, 20709–20716.

Kang, Y., Chen, C.-R., and Massagué, J. (2003). A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol. Cell 11, 915–926.

Kato, Y., Habas, R., Katsuyama, Y., Naar, A.M., and He, X. (2002). A component of the ARC/Mediator complex required for TGFβ/Nodal signalling. Nature 418, 641–646.

Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., and Wrana, J.L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. Mol. Cell 6, 1365–1375.

Kawabata, M., Inoue, H., Hanyu, A., Imamura, T., and Miyazono, K. (1998). Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. EMBO J. 17, 4056–4065.

Kim, J., Johnson, K., Chen, H.J., Carroll, S., and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. Nature 388, 304–308.

Kirsch, T., Sebald, W., and Dreyer, M.K. (2000). Crystal structure of the BMP-2-BRIA ectodomain complex. Nat. Struct. Biol. 7, 492–496.

Kraulis, P.J. (1991). Molscript: a program to produce both detailed and schematic plots of protein structures. J. Appl. Crystallogr. 24, 946–950.

Kretzschmar, M., Liu, F., Hata, A., Doody, J., and Massagué, J. (1997). The TGF-β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev. 11, 984–995.

Kretzschmar, M., Doody, J., Timokhina, I., and Massagué, J. (1999). A mechanism of repression of TGF-β/Smad signaling by oncogenic Ras. Genes Dev. 13, 804–816.

Kurisaki, A., Kose, S., Yoneda, Y., Heldin, C.-H., and Moustakas, A. (2001). Transforming growth factor-β induces nuclear import of Smad3 in an importin-β1 and Ran-dependent manner. Mol. Biol. Cell 12, 1079–1091.

Labbe, E., Silvestri, C., Hoodless, P.A., Wrana, J.L., and Attisano, L. (1998). Smad2 and Smad3 positively and negatively regulate TGF-β-dependent transcription through the forkhead DNA-binding protein FAST2. Mol. Cell 2, 109–120.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001). Initial sequencing and analysis of the human genome. Nature 409, 860–921.

Lewis, K.A., Gray, P.C., Blount, A.L., MacConell, L.A., Wiater, E., Bilezikjian, L.M., and Vale, W. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature 404, 411–414.

Lim, D.A., Tramontin, A.D., Trevejo, J.M., Herrera, D.G., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (2000). Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 28, 713–726.

Lin, X., Liang, M., and Feng, X.-H. (2000). Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in TGF-β signaling. J. Biol. Chem. 275, 36818–36822.

Liu, F., Pouponnot, C., and Massagué, J. (1997). Dual role of the Smad4/DPC4 tumor suppressor in TGF-β-inducible transcriptional complexes. Genes Dev. 11, 3157–3167.

Liu, X., Sun, Y., Weinberg, R.A., and Lodish, H.F. (2001). Ski/Sno and TGF-β signaling. Cytokine Growth Factor Rev. 12, 1–8.

Lo, R.S., and Massagué, J. (1999). Ubiquitin-dependent degradation of TGF-β-activated Smad2. Nat. Cell Biol. 1, 472–478.

Lo, R.S., Chen, Y.-G., Shi, Y., Pavletich, N.P., and Massagué, J. (1998). The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-β receptors. EMBO J. 17, 996–1005.

Lo, R.S., Wotton, D., and Massagué, J. (2001). Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. EMBO J. 20, 128–136.

Lu, Z., Murray, J.T., Luo, W., Li, H., Wu, X., Xu, H., Backer, J.M., and Chen, Y.G. (2002). Transforming growth factor β activates Smad2 in the absence of receptor endocytosis. J. Biol. Chem. 277, 29363–29368.

Luo, K., Stroschein, S.L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999). The Ski oncoprotein interacts with the Smad proteins to repress TGF-β signaling. Genes Dev. 13, 2196–2206.

Macias-Silva, M., Abdollah, S., Hoodless, P.A., Pirone, R., Attisano, L., and Wrana, J.L. (1996). MADR2 is a substrate of the TGF-β receptor and its phosphorylation is required for nuclear accumulation and signalling. Cell 87, 1215–1224.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. Science 298, 1912–1934.

Marchuk, D.A. (1998). Genetic abnormalities in hereditary hemorrhagic telangiectasia. Curr. Opin. Hematol. 5, 332–338.

Marlow, M.S., Brown, C.B., Barnett, J.V., and Krezel, A.M. (2003). Solution structure of the chick TGF-β type II receptor ligand-binding domain. J. Mol. Biol. 326, 989–997.

Massagué, J. (1998). TGF-β signal transduction. Annu. Rev. Biochem. 67, 753–791.

Massagué, J. (2000). How cells read TGF-β signals. Nat. Rev. Mol. Cell Biol. 1, 169–178.

Massagué, J., and Chen, Y.-G. (2000). Controlling TGF-β signaling. Genes Dev. 14, 627–644.

Massagué, J., and Wotton, D. (2000). Transcriptional control by the TGF-β/Smad signaling system. EMBO J. 19, 1745–1754.

Massagué, J., Blain, S.W., and Lo, R.S. (2000). TGF-β Signaling in growth control, cancer, and heritable disorders. Cell 103, 295–309.

Mattaj, I.W., and Englmeier, L. (1998). Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. 67, 265–306.

Mirura, S., Takeshita, T., Asao, H., Kimura, Y., Murata, K., Sasaki, Y., Hanai, J.I., Beppu, H., Tsukazaki, T., Wrana, J.L., et al. (2000). Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol. Cell. Biol. 20, 9346–9355.

Mittl, P.R., Priestle, J.P., Cox, D.A., McMaster, G., Cerletti, N., and Grutter, M.G. (1996). The crystal structure of TGF-beta 3 and comparison to TGF-β2: implications for receptor binding. Protein Sci. 5, 1261–1271.

Nishihara, A., Watabe, T., Imamura, T., and Miyazono, K. (2002). Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. Mol. Biol. Cell 13, 3055–3063.

Onichtchouk, D., Chen, Y.G., Dosch, R., Gawantka, V., Delius, H., Massagué, J., and Niehrs, C. (1999). Silencing of TGF-β signalling by the pseudoreceptor BAMBI. Nature 401, 480–485.

Patterson, G.I., and Padgett, R.W. (2000). TGF-β-related pathways. Roles in Caenorhabditis elegans development. Trends Genet. 16, 27–33.

Petritsch, C., Beug, H., Balmain, A., and Oft, M. (2000). TGF-β inhibits p70 S6 kinase via protein phosphatase 2A to induce G1 arrest. Genes Dev. 14, 3093–3101.

Pierreux, C.E., Nicolas, F.J., and Hill, C.S. (2000). Transforming growth factor β-independent shuttling of Smad4 between the cytoplasm and nucleus. Mol. Biol. Cell 20, 9041–9054.

Qin, B.Y., Chacko, B.M., Lam, S.S., de Caestecker, M.P., Correia, J.J., and Lin, K. (2001). Structural basis of Smad1 activation by receptor kinase phosphorylation. Mol. Cell 8, 1303–1312.

Randall, R.A., Germain, S., Inman, G.S., Bates, P.A., and Hill, C.S. (2002). Different Smad2 partners bind a common hydrophobic pocket in Smad2 via a defined proline-rich motif. EMBO J. 21, 145–156.

Rosa, F.M. (2002). Cripto, a multifunctional partner in signaling: molecular forms and activities. Sci. STKE (158), pe47.

Saha, D., Datta, P.K., and Beauchamp, R.D. (2001). Oncogenic ras represses transforming growth factor-β/Smad signaling by degrading tumor suppressor Smad4. J. Biol. Chem. 276, 29531–29537.

Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., and Gelbart, W.M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics 139, 1347–1358.

Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., and Massagué, J. (2001). TGF-β influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat. Cell Biol. 3, 400–408.

Shen, M.M., and Schier, A.F. (2000). The EGF-CFC gene family in vertebrate development. Trends Genet. 16, 303–309.

Shi, Y. (2001). Structural insights on Smad function in TGF-β signaling. Bioessays 23, 223–232.

Shi, Y., Wang, Y.-F., Jayaraman, L., Yang, H., Massagué, J., and Pavletich, N.P. (1998). Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-β signaling. Cell 94, 585–594.

Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin, C.H. (1997). Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. J. Biol. Chem. 272, 28107–28115.

Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massagué, J., Hanel, F., and Eilers, M. (2001). Repression of p15INK4b expression by Myc through association with Miz-1. Nat. Cell Biol. 3, 392–399.

Stroschein, S.L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999). Negative feedback regulation of TGF-β signaling by the SnoN oncoprotein. Science 286, 771–774.

Stroschein, S.L., Bonni, S., Wrana, J.L., and Luo, K. (2001). Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. Genes Dev. 15, 2822–2836.

Sun, P.D., and Davies, D.R. (1995). The cysteine-knot growth-factor superfamily. Annu. Rev. Biophys. Biomol. Struct. 24, 269–291.

Suzuki, C., Murakami, G., Fukuchi, M., Shimanuki, T., Shikauchi, Y., Imamura, T., and Miyazono, K. (2002). Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. J. Biol. Chem. 277, 39919–39925.

Tajima, Y., Goto, K., Yoshida, M., Shinomiya, K., Sekimoto, T.,

Yoneda, Y., Miyazono, K., and Imamura, T. (2003). Chromosomal region maitenance 1 (CRM1)-dependent nuclear export of Smad ubiquitin regulatory factor 1 (Smurf1) is essential for negative regulation of transforming growth factor-β signaling by Smad7. J. Biol. Chem. *278*, 10716–10721.

Tang, Y., Katuri, V., Dillner, A., Mishra, B., Deng, C.X., and Mishra, L. (2003). Disruption of transforming growth factor-β signaling in ELF β-spectrin–deficient mice. Science *299*, 574–577.

ten Dijke, P., Miyazono, K., and Heldin, C.-H. (2000). Signaling inputs converge on nuclear effectors in TGF-β signaling. Trends Biochem. Sci. *25*, 64–70.

ten Dijke, P., Goumans, M.-J., Itoh, F., and Itoh, S. (2002). Regulation of Cell Proliferation by Smad Proteins. J. Cell. Physiol. *191*, 1–16.

Thompson, T.B., Woodruff, T.K., and Jardetzky, T.S. (2003). Structures of an ActRIIB:activin A complex reveal a novel binding mode for TGF-β ligand:receptor interactions. EMBO J. *22*, 1555–1566.

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., and Wrana, J.L. (1998). SARA, a FYVE Domain Protein that Recruits Smad2 to the TGF-β Receptor. Cell *95*, 779–791.

Ulloa, L., Doody, J., and Massagué, J. (1999). Inhibition of transforming growth factor-β/SMAD signalling by the interferon-gamma/STAT pathway. Nature *297*, 710–713.

Wan, Y., Liu, X., and Kirschner, M.W. (2001). The anaphase-promoting complex mediates TGF-β signaling by targeting SnoN for destruction. Mol. Cell *8*, 1027–1039.

Wang, W., Mariani, F.V., Harland, R.M., and Luo, K. (2000). Ski represses bone morphogenetic protein signaling in Xenopus and mammalian cells. Proc. Natl. Acad. Sci. USA *97*, 14394–14399.

Watanabe, M., Masuyama, N., Fukuda, M., and Nishida, E. (2000). Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. EMBO Rep. *1*, 176–182.

Wicks, S.J., Lui, S., Abdel-Wahab, N., Mason, R.M., and Chantry, A. (2000). Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. Mol. Cell. Biol. *20*, 8103–8111.

Wotton, D., Lo, R.S., Lee, S., and Massagué, J. (1999a). A Smad transcriptional corepressor. Cell *97*, 29–39.

Wotton, D., Lo, R.S., Swaby, L.A., and Massagué, J. (1999b). Multiple modes of repression by the Smad transcriptional corepressor TGIF. J. Biol. Chem. *274*, 37105–37110.

Wotton, D., Knoepfler, P.S., Laherty, C.D., Eisenman, R.N., and Massagué, J. (2001). The Smad transcriptional corepressor TGIF recruits mSin3. Cell Growth Differ. *12*, 457–463.

Wu, G., Chen, Y.-G., Ozdamar, B., Gyuricza, C.A., Chong, P.A., Wrana, J.L., Massagué, J., and Shi, Y. (2000). Structural basis of Smad2 recognition by the Smad anchor for receptor activation. Science *287*, 92–97.

Wu, J.-W., Fairman, R., Penry, J., and Shi, Y. (2001a). Formation of a stable heterodimer between Smad2 and Smad4. J. Biol. Chem. *276*, 20688–20694.

Wu, J.-W., Hu, M., Chai, J., Seoane, J., Huse, M., Li, C., Rigotti, D.J., Kyin, S., Muir, T.W., Fairman, R., et al. (2001b). Crystal structure of a phosphorylated Smad2: recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-β signaling. Mol. Cell *8*, 1277–1289.

Wu, J.-W., Krawitz, A.R., Chai, J., Li, W., Zhang, F., Luo, K., and Shi, Y. (2002). Structural mechanism of Smad4 recognition by the nuclear oncoprotein Ski: insights on Ski-mediated repression of TGF-β signaling. Cell *111*, 357–367.

Xiao, Z., Liu, X., Henis, Y.I., and Lodish, H.F. (2000a). A distinct nuclear localization signal in the N terminus of Smad3 determines its ligand-induced nuclear translocation. Proc. Natl. Acad. Sci. USA *97*, 7853–7858.

Xiao, Z., Liu, X., and Lodish, H.F. (2000b). Importin β mediates nuclear translocation of Smad3. J. Biol. Chem. *275*, 23425–23428.

Xiao, Z., Watson, N., Rodriguez, C., and Lodish, H.F. (2001). Nucleocytoplasmic shuttling of Smad1 conferred by its nuclear localization and nuclear export signal. J. Biol. Chem. *276*, 39404–39410.

Xiao, Z., Latek, R., and Lodish, H.F. (2003). An extended bipartite nuclear localization signal in Smad4 is required for its nuclear import and transcriptional activity. Oncogene *22*, 1057–1069.

Xu, L., Chen, Y.-G., and Massagué, J. (2000). Smad2 nuclear import function masked by SARA and unmasked by TGFβ-dependent phosphorylation. Nat. Cell Biol. *2*, 559–562.

Xu, L., Kang, Y., Col, S., and Massagué, J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGF-β signaling complexes in the cytoplasm and nucleus. Mol. Cell *10*, 271–282.

Yagi, K., Goto, D., Hamamoto, T., Takenoshita, S., Kato, M., and Miyazono, K. (1999). Alternatively spliced variant of Smad2 lacking exon *3*. J. Biol. Chem. *274*, 703–709.

Yingling, J.M., Datto, M.B., Wong, C., Frederick, J.P., Liberati, N.T., and Wang, X.-F. (1997). Tumor suppressor Smad4 is a transforming growth factor β-inducible DNA binding protein. Mol. Cell. Biol. *17*, 7019–7028.

Yu, L., Hebert, M.C., and Zhang, Y.E. (2002). TGF-β receptor-activated p38 MAP kinase mediates Smad-independent TGF-β responses. EMBO J. *21*, 3749–3759.

Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and Kern, S.E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. Mol. Cell *1*, 611–617.

Zhang, Y., Chang, C., Gehling, D.J., Hemmati-Brivanlou, A., and Derynck, R. (2001). Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc. Natl. Acad. Sci. USA *98*, 974–979.

Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L., and Thomsen, G.H. (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature *400*, 687–693.